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HAG3, a Histone Acetyltransferase, affects UV-B Responses by negatively regulating the Expression of DNA Repair Enzymes and Sunscreen Content in *Arabidopsis thaliana*

Running head: GNAT family acetyltransferases in UV-B responses

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Abbreviations: CBP, CREB-binding protein; ELP, ELongator complex Protein; GNAT, GCN5-related N-acetyltransferase; GCN, General Control Nonderepressible protein; HAG, Histone acetyltransferase in the GNAT family; HAT, histone acetyltransferase; MS, Murashige and Skoog; RT–PCR; MYST, MOZ, Ybt2, Sas2, Tip60-like family proteins; reverse transcription–PCR; UTR, untranslated region; TAF, TATA binding proteing associated factor; UV, ultraviolet.

ABSTRACT

Histone acetylation is regulated by histone acetyltransferases and deacetylases. In Arabidopsis, there are 12 histone acetyltransferases and 18 deacetylases, histone acetyltransferases are organized in 4 families: the GNAT/HAG, the MYST, the p300/CBP and the TAFII250 families. Previously, we demonstrated that Arabidopsis mutants in the two members of the MYST acetyltransferase family show increased DNA damage after UV-B irradiation. To further investigate the role of other histone acetyltransferases in UV-B responses, a putative role of enzymes of the GNAT family, HAG1, HAG2 and HAG3, was analyzed. *HAG* transcripts are not UV-B regulated; however, *hag3* RNAi transgenic plants show a lower inhibition of leaf and root growth by UV-B, higher levels of UV-B absorbing compounds and less UV-B induced DNA damage than Ws plants, while *hag1* RNAi transgenic plants and *hag2* mutants do not show significant differences with WT plants. Transcripts for UV-B regulated genes are highly expressed under control conditions in the absence of UV-B in *hag3* RNAi transgenic plants, suggesting that the higher UV-B tolerance may be due to increased levels proteins that participate in UV-B responses. Together, our data provide evidence that HAG3, directly or indirectly, participate in UV-B induced-DNA damage repair and signaling.

Keywords*:* DNA damage; GNAT familiy; histone acetyltransferases; UV-B damage.

Introduction

Eukaryotic DNA is compacted into chromatin, which basic unit is the nucleosome. Each nucleosome core particle is composed by 147 bp of DNA wrapped around a histone octamer consisting of two molecules of histones H2A, H2B, H3, and H4 (for a review, see Pfluger and Wagner 2007). This compact structure is a barrier to proteins that participate in DNA metabolism, such as transcription, replication, and DNA recombination and repair; therefore chromatin must be restructured to allow these processes to occur. Three different processes regulate chromatin accessibility: covalent modification of histones, chromatin remodeling activities that alter histone–DNA interaction and DNA methylation of cytosines (Pfluger and Wagner 2007; Vaillant and Paszkowski 2007; Eberharter and Becker 2002). Histone post-translational modifications include acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and poly-ADP-ribosylation (Kouzarides 2007). Hyperacetylation of histone lysines is a characteristic of actively transcribed genes, while transcriptionally inactive DNA is typically associated with hypoacetylated histones (Hebbes et al. 1988; Richards and Elgin 2002; Sterner and Berger 2000). When lysines at the amino-terminal histone tails are acetylated, histone–DNA interactions are altered to make the DNA more accessible (Kouzarides 2007). Thus, histone acetylation and deacetylation are essential for the regulation of plant gene expression (Fuchs et al. 2006; Loidl 2004; Lusser et al. 2001; Richards and Elgin 2002). Also, the disruption of the nucleosome–DNA interactions mediated by histone acetylation can alter DNA repair rates. For example, we previously found that maize and Arabidopsis plants treated with an inhibitor of histone acetyltransferases, curcumin, previous to a UV-B treatment show deficiencies in DNA repair (Campi et al. 2012). Moreover, mass spectrometry analysis of post-translational histone modifications in maize demonstrated that UV-B exposed plants exhibited greater acetylation of N-terminal H3 and H4 tails (Casati et al. 2008).

Histone acetylation is regulated by the activities of histone acetyltransferases and histone deacetylases. In Arabidopsis, there are 12 histone acetyltransferases and 18 deacetylases (Pandey et al. 2002). Histone acetyltransferases are organized in four families based on sequence homology and/or mode of action: the GNAT family (from GCN5-related N-acetyltransferase; HAG1, HAG2 and HAG3), the MYST family (from MOZ, Ybt2, Sas2, Tip60-like; HAM1 and HAM2), the p300/CBP family (HAC1, HAC2, HAC4, HAC5 and HAC12), and the TAFII250 family (HAF1 and HAF2; Pandey et al. 2002). In particular, the GNAT family comprises three subfamilies in higher eukaryotes, designated GCN5 (General Control Nonderepressible

protein5), ELP3 (a transcriptional ELongator complex Protein), and HAT1. In the Arabidopsis genome, a single homolog of each of the GCN5 (HAG1/AtGCN5), ELP3 (HAG3), and HAT1 (HAG2) proteins has been identified (Pandey et al. 2002). HAG1, HAG2 and HAG3 are defined by the presence of a Histone Acetyltransferase (HAT) domain (Supplementary Fig. S1) that is comprised of four motifs A-D (Sterner and Berger 2000). Besides this domain, the three proteins show low sequence similarity; HAG1 has also a bromodomain, and HAG3 has a radical S-adenosylmethionine binding domain (Supplementary Fig. S1). These three enzymes have been shown to acetylate histones but in different Lys residues; for instance, HAG1 specifically acetylates H3K14 (Earley et al. 2007), HAG2 acetylates H4K12 (Earley et al. 2006), while *hag3* mutants are deficient in H3K56Ac and H4K5Ac (Xu et al. 2012). Moreover, these three proteins were reported to have different functions in Arabidopsis. HAG1 and its associated adaptor proteins (similar to yeast SAGA complex; Sterner and Berger 2000) have been shown to participate in plant development, and in light and cold regulated gene expression (Servet et al. 2010); while HAG3 is a subunit of the Elongator complex, mutations in different subunits of this complex result in hypersensitivity to abscisic acid, resistance to oxidative stress, severely aberrant auxin phenotypes, disease susceptibility, and altered cell cycle progression (Nelissen et al. 2005; Chen et al. 2006 ; Zhou et al. 2009, Defraia et al. 2010; Xu et al. 2012). Although not much is known about the role of HAG2 *in vivo*, this protein is regulated by E2F transcription factors that induce the transcription of genes required for cell cycle progression and DNA replication (Ramirez-Parra et al. 2003; Vandepoele *et al.* 2005).

Plants are constantly exposed to different environmental conditions. Because plants require light for photosynthesis, they are also exposed to fluctuating solar ultraviolet-B radiation (UV-B 290-315 nm) and high UV-B irradiation causes direct damage to DNA, proteins, lipids, and RNA (Britt 1996; Gerhard et al. 1999; Casati and Walbot 2004). In particular, absorption of UV-B by DNA induces the formation of covalent bonds between adjacent pyrimidines, producing cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) (Friedberg et al. 1995). These lesions disrupt base pairing and block DNA metabolism if photoproducts persist, or result in mutations if photoproducts are bypassed by error-prone DNA polymerases (Britt 1996). Thus, such lesions must be repaired to maintain genome integrity; and, as plants lack a reserved germ line, to avoid mutations occurring in somatic cells to be transmitted to their progeny. Therefore, plants have evolved different DNA repair systems to remove or tolerate DNA lesions, including chromatin remodeling activities (Bray and West 2005; Hays 2002; Kimura and Sakaguchi 2006). In Arabidopsis, Takeda et al. (2004) demonstrated that BRU1, a chromatin-

related gene, participates in DNA repair; while Shaked et al. (2006) showed that 14 of the 40 Arabidopsis SWI2/SNF2 gene family members also had a role in DNA damage response against gamma or UV-C radiation and recombination. In addition, we have also shown that maize and Arabidopsis plants with decreased levels of chromatin remodeling activities showed increased CPD accumulation after UV-B exposure (Campi et al. 2012, Questa et al. 2013). On the other hand, chromatin remodeling is also a key process during transcription of genes regulated by UV-B (Casati et al. 2006 and 2008), suggesting that the role of chromatin proteins in DNA repair can be direct, making DNA more accessible to DNA repair enzymes, or indirect, regulating the expression of these enzymes.

Previously, we demonstrated that Arabidopsis mutants in *HAM1* and *HAM2,* the two members of the MYST acetyltransferase family in this plant species, showed increased DNA damage after a 4h-UV-B-treatment, suggesting that the role of these proteins in DNA damage repair has been conserved through evolution, as in humans, TIP60, which is homologous to the Arabidopsis HAM proteins, has important functions during DNA repair, transactivating genes in response to DNA damage, and more importantly, acetylating H4 when DNA is damaged (Squatrito et al. 2006). Together, several investigations demonstrated that chromatin remodeling, and histone acetylation in particular, are essential during DNA repair and UV-B responses (Casati et al. 2008; Campi et al. 2013). To further investigate the role of histone acetyltransferases in UV-B responses and DNA repair, a putative role of enzymes of the GNAT family was analyzed in Arabidopsis. We found that HAG acetyltrasferases are not regulated by UV-B radiation in WT plants. However, using plants with decreased transcript levels of *HAG1, HAG2* and *HAG3*, we demonstrated that *hag3* RNAi plants showed lower inhibition of leaf and root growth by UV-B and higher levels of UV-B absorbing compounds, while *hag1* and *hag2* plants did not show significant differences with WT plants. Also, *hag3* plants showed less CPDs than Ws plants after a UV-B treatment, while plants deficient in HAG1 and HAG2 had similar DNA damage than WT plants. Transcripts for two DNA repair enzymes were highly expressed under control conditions in the absence of UV-B in *hag3* plants, suggesting that the lower accumulation of photoproducts by UV-B may be due to increased DNA repair in these transgenic plants. Finally, we here demonstrate that several genes that are upregulated by UV-B in WT plants showed increased expression levels in *hag3* plants, demonstrating crucial roles for HAG3 in UV-B signaling. Together, our data provide evidence that HAG3, directly or indirectly, participate in UV-B induced-DNA damage repair and signaling.

Results

UV-B regulation of *HAG1*, *HAG2* and *HAG3*, and mutant and transgenic plant analysis

Chromatin remodeling and histone acetylation in particular has previously been shown to be crucial for UV-B damage repair in plants (Campi et al. 2012; Lario et al. 2013: Questa et al. 2013). Therefore, we sought to determine if, besides the already described role of HAM1 and HAM2 in DNA repair in Arabidopsis, other histone acetyltranferases in this species participate in UV-B damage repair. In this manuscript, we focused in the three members of the GNAT family

We have previously reported that *HAM1* and *HAM2* are upregulated by UV-B (Campi et al. 2013); thus, we investigated if the histone acetyltransferases of the GNAT family were similarly regulated. WT plants of the Columbia-0 ecotype grown in the absence of UV-B were exposed under UV-B lamps for 4h (2 $W.m^{-2}$) in a growth chamber, this is the UV-B intensity in Rosario, Argentina, at noon during summer time. After the treatment, leaf tissue from 4-week old plants was collected for RNA extraction and qRT-PCR analysis. Interestingly, none of the transcripts were regulated by UV-B (Supplementary Fig. S2). To further investigate the role of these 3 proteins in UV-B responses, *A. thaliana* lines defective in *HAG1, HAG2* and *HAG3* expression were identified in the SALK and RNAi knockdown from the Functional Genomics of Chromatin: Global Control of Plant Gene Expression collections (http://www.chromdb.org/rnai/rnai_lines_info.html). For *hag1* and *hag3*, several independent RNAi lines were obtained from the Plant Chromatin collection (see Material and Methods); although RNAi plants may have off-targets, both *hag1* and *hag3* RNAi transgenic lines had a significant reduction of *HAG1* and *HAG3* transcripts, respectively, in comparison to WT plants (Supplementary Fig. S3). For the *HAG2* gene, two T-DNA insertional lines, SALK_051832 and SALK_152796, both with an insertion in the second intron, were identified (Supplementary Fig. S3) by a PCR screen using gene-specific primers and one specific primer for the T-DNA left border (Table 1). The consequences of the insertion of the T-DNA in the second intron of the *HAG2* gene in both *hag2* mutants was confirmed by qRT-PCR on plants homozygous for the mutant allele (Supplementary Fig. S3).

Physiological analysis of plants with decreased levels of *HAG1*, *HAG2* and *HAG3*

Depending on the dose and treatment condition, UV-B radiation produces the reduction of the biomass in plants (Bornman and Teramura 1993). We have previously demonstrated that a UV-B treatment for 4 h with an intensity of 2 W m^{-2} decreased the rosette area in UV-B treated Arabidopsis plants; and that the difference in rosette size between treated and untreated plants persisted 10 d after the treatment (Casadevall et al. 2013). We also found that the leaf area of leaf #5, which was emerging and proliferating at the moment of the UV-B treatment, was significantly reduced in UV-B treated plants. This was because the leaves had a decreased number of cells, while the average cell area was similar in treated and untreated leaves, demonstrating that after a single UV-B treatment in developing leaves with proliferating cells, UV-B inhibits cell proliferation (Casadevall et al. 2013). Therefore, to evaluate if the effect of UV-B in plant growth inhibition requires any or some HAG activities, we analyzed the effect of UV-B in growth inhibition using the Arabidopsis plants deficient in each HAG proteins. Under normal conditions in the absence of UV-B, all plants with decreased levels of *HAG1*, *HAG2* and *HAG3* were smaller than wild type in the Ws or Col 0 backgrounds (Fig. 1), demonstrating that the three proteins have a role in plant development and proliferation, as it was already reported (Servet et al. 2010, Vandepoele et al. 2005, Nelissen et al. 2005). However, while WT and plants with decreased levels of *HAG1* and *HAG2* exhibited a similar decrease in rosette area after a single UV-B treatment for 4 h; the *hag3* transgenic line had a significant lower inhibition of plant growth than Ws plants (Fig. 1). In this way, after the UV-B treatment, both *hag3* and WT plants showed a similar rosette area (Fig. 1). Moreover, while in *hag3* control plants, leaf #5 (which was proliferating at the moment of the UV-B treatment) was smaller than WT leaf #5; in UV-B irradiated plants, average leaf #5 area in *hag3* plants was similar to that of Ws plants (Fig. 2A). Interestingly, in control plants, *hag3* leaf #5 was smaller because it had smaller cells, as the number of cells in leaf #5 from *hag3* and WT plants was similar, in contrast to previous reports where *hag3* mutants showed inhibition of cell proliferation (Nelissen et al. 2005, Fig. 2B). However, after the UV-B treatment, although both *hag3* and WT plants had a similar inhibition of cell proliferation (Fig. 2B), *hag3* mutants showed an increase in cell area that was not observed in Ws leaf #5 cells (Fig. 2C), suggesting that a compensation effect is taking place in the transgenic lines after the UV-B treatment. In this way, leaf #5 from the *hag3* RNAi plants, which already has a cell proliferation defect, can reach a final leaf size and it is not further reduced. Interestingly, levels of the miR396 and GRFs, which we have previously shown to participate in the inhibition of cell proliferation by UV-B (Casadevall et al. 2013) were not affected in *hag3* plants, neither under control nor after UV-B exposure (data not shown), suggesting that HAG3 participates in a different developmental pathway.

In addition, UV-B sensitivity in the three lines was analyzed by inhibition of primary root elongation assays (Tong et al. 2008). Fig. 3 shows that one day after the end of the UV-B treatment, while Ws plants showed a significant decrease in primary root elongation compared to untreated plants, *hag3* plants showed only a minor inhibition. This difference persisted 4 days after the treatment. However, *hag1* and *hag2* plants showed a similar decrease in primary root growth as WT plants (Fig. 3). Together, these results suggest that *hag3* plants are less sensitive to growth inhibition by UV-B radiation than WT plants; while *hag1* and *hag2* plants are similarly affected by this radiation as WT plants.

In plants, the first line of defense when exposed to UV-B is the synthesis of protective pigments like flavonoids and UV-B absorbing pigments. Thus, we then investigated the effect of UV-B on UV sunscreens (Li et al. 1993; Landry et al. 1995; Ormrod et al. 1995). After a 4h-UV-B treatment, the concentration of these molecules was 1.3-fold higher than under control conditions in Ws and Col0 plants (Fig. 4). Similar increases were observed for *hag1* and *hag2* plants (Fig. 4 D-F), despite that *hag2* mutants showed a lower content of UV-B absorbing compounds under both conditions (Fig. 4B). On the contrary, plants with decreased levels of *HAG3* transcripts had altered accumulation of UV sunscreen photoprotectors. *hag3* plants showed a significantly higher basal levels of these pigments than WT plants before the UV-B treatment (2.2-fold increase, Fig. 4C), which were elevated and similar as levels after the UV-B treatment. This was also true for anthocyanin accumulation (Fig. 4D), as it was previously reported for different Elongator mutants (Zhou et al. 2009).

HAG3 but not *HAG1* or *HAG2* deficient plants show decreased UV-B induced DNA damage

To test if HAG proteins participate in UV-B induced DNA repair as previously reported for HAM acetyltransferases, we grew *A. thaliana* WT plants and plants deficient in the expression of *HAG1*, *HAG2* and *HAG3* in the growth chamber in the absence of UV-B for 4-weeks, and plants were then exposed to UV-B for 4h (2 $W.m^{-2}$). As a control, different sets of plants were irradiated with the same lamps covered with a polyester plastic that absorbs UV-B. Leaf samples from control and treated plants were collected immediately after the end of the UV-B treatment. DNA was extracted and CPD abundance was assayed after the treatments. Fig. 5 shows that UV-B induced a CPD accumulation in all lines, including WT plants. Comparison of CPD accumulation in samples from WT and *HAG1*, *HAG2* and *HAG3* deficient plants under control conditions in the absence of UV-B showed that the steady state levels of CPDs were

similar and very low (~200 optical density (IOD) in all samples). However, after 4h of UV-B exposure, while similar amounts of CPDs accumulated in *hag1* and *hag2* lines and in the WT plants (Fig. 5 A and B); *hag3* plants showed a lower accumulation of CPDs (Fig. 5C). Therefore, consistent with the lack of UV-B sensitivity observed in the growth inhibition assays, *hag3* plants accumulate lower levels of CPDs than WT plants.

hag plants have altered levels of UV-B regulated transcripts

The evidence of a role of HAG3 in UV-B damage repair prompted us to investigate its involvement in the regulation of the expression of DNA repair genes. UV-B-induced DNA damage repair is accomplished by two main pathways: nucleotide excision repair (NER) and photoreactivation (PR). Therefore, we measured the transcript levels of two selected NER and PR genes before and after UV-B exposure. We evaluated the expression of *UVR2*, encoding a CPD photolyase (At1g12370); and *UVR7,* encoding ERCC1, a DNA excision repair protein (At3g05210). Fig. 6C shows that, although both genes are up-regulated by UV-B radiation in WT plants after the treatment; in *hag3* RNAi plants these genes are highly expressed in control conditions in the absence of UV-B. In previous studies using different mutants that are deficient in homologous recombination and repair of damaged DNA with methylmetane sulphonate, such as *abo4* (a mutant in the DNA pol ε, Yin et al. 2009), *rfc1* (a mutant in the DNA replication factor C1; Liu et al. 2010a) and *pol*α (a mutant in the DNA pol α, Liu et al. 2010b), and *ddm1* (Questa et al. 2013), DNA repair transcripts were highly and constitutively expressed, suggesting that in these mutants DNA repair-related genes were spontaneously induced. We hypothesize that a similar situation occurs in *hag3* plants. Thus, the lower accumulation of CPDs in *hag3* mutants may be a result of higher basal levels of DNA repair enzymes.

On the other hand, we analyzed the expression of other genes that are upregulated by UV-B radiation in Arabidopsis (Ulm et al. 2004). HY5 is a bZIP transcription factor that is required for UV-B-mediated regulation of many genes, while chalcone synthase (CHS) and flavanone 3 hydroxylase (F3H) participate in the biosynthesis of flavonoids. In our experiments, all these transcripts were induced by UV-B in the Col0 and Ws backgrounds (Fig. 6); however, these transcripts showed increased basal expression levels in control conditions in the absence of UV-B in *hag3* plants compared to Ws (Fig. 6C), as measured for the DNA repair enzymes. Higher expression of *CHS* and *F3H* in *hag3* plants correlates

with high accumulation of UV-B absorbing compounds in control conditions shown in Fig. 4. Collectively, the increased levels of transcripts of proteins that participate in UV-B responses suggest that *hag3* plants are better adapted to deal with increased levels of UV-B radiation, demonstrating that HAG3 has a key function in regulating UV-B responses in Arabidopsis.

On the other hand, although the levels of some UV-B regulated transcripts in *hag1* and *hag2* plants were similar as those measured in WT plants both under control conditions and after UV-B exposure, some transcripts show a different pattern of expression in these lines in comparison to WT plants (Fig. 6 A an B). For example, a differential expression of UVR7, CHS and F3H was measured when comparing Ws and *hag1* plants; while UVR2, HY5 and F3H were differentially expressed in Col0 and *hag2* plants. So, it is possible that HAG1 and HAG2 may also participate in some aspects of UV-B responses in Arabidopsis.

Discussion

In maize and Arabidopsis, chromatin remodeling and histone acetylation in particular have been implicated in UV-B responses and DNA damage repair (Casati et al. 2006; Casati and Walbot, 2008; Campi et al. 2012; Qüesta et al. 2013). Transgenic maize and Arabidopsis plants knockdown for chromatin remodeling genes and Arabidopsis mutants were found to be acutely sensitive to UV-B, and these plants were deficient in UV-B damaged DNA repair (Campi et al. 2012; Qüesta et al. 2013). In addition, maize and Arabidopsis plants with lower expression of chromatin-associated genes also exhibited altered UV-B regulation of selected genes (Casati et al. 2006; Qüesta et al. 2013). In particular, because histone H3 and H4 acetylation is significantly increased by UV-B (Casati et al. 2008), we previously analyzed the effect of histone acetylation on DNA repair, and we demonstrated that when plants were pre-treated with curcumin, a histone acetylase inhibitor, DNA repair was impaired (Campi et al. 2012). Moreover, Arabidopsis mutants in *HAM1* and *HAM2*, two histone acetyltransferases from the MYST family, showed increased DNA damage after a 4h-UV-B-treatement. Therefore, the role of MYST family acetyltransferases in DNA damage repair seems to be conserved; as the human homologue to Arabidopsis HAM proteins, TIP60, has demonstrated roles during DNA repair, transactivating genes in response of DNA damage and acetylating H4 when DNA is damaged (Squatrito et al. 2006). In Arabidopsis, there are 12 different acetyltranferases; these have been mostly linked to transcriptional

regulation. Thus, in this work, to further investigate the role of histone acetylation in UV-B responses, a putative role of HAG1, HAG2 and HAG3, acetyltransferases of the GNAT family, was analyzed in Arabidopsis.

HAG1/GCN5 plays a role in the regulation of cold tolerance, floral development, embryonic cell-fate patterning, and light responsiveness (Stockinger et al. 2001; Vlachonasios et al. 2003; Bertrand et al. 2003, Benhamed et al. 2006; Long et al. 2006); the role of HAG2 has not been previously described, but it is regulated by the E2F transcription factors that control cell cycle progression and DNA replication (Ramirez-Parra et al. 2003; Vandepoele et al. 2005); while HAG3/ELO3 is a subunit of the Elongator complex. Elongator is a histone acetyl-transferase complex that directly interacts with RNA polymerase-II in transcriptional elongation in different eukaryotes, including plants (Chen et al. 2006; Otero et al. 1999). In all species, Elongator consists of two subcomplexes: the core subcomplex with subunits ELP1, 2 and 3, and the accessory subcomplex with subunits ELP4, 5 and 6 (Krogan and Greenblatt 2001). This complex have been suggested to participate in different cellular pathways, such as in RNAPII-mediated transcription elongation through the acetylation of histone H3 and H4, the modification of certain tRNAs and the acetylation of α-tubulin (Otero et al. 1999; Fellows et al. 2000; Jablonowski et al. 2001; Huang et al. 2005; Esberg et al. 2006; Svejstrup 2007; Creppe et al. 2009). Elongator has also been shown to be involved in DNA replication and repair, and in gene silencing (for a review, see Xu et al. 2014). The composition of the Elongator complex is highly conserved in eukaryotes, protein homologs of various subunits have been identified in fungi, plants and animals, including humans. Elongator mutants (*elo*) in plants have pleiotropic phenotypes including defects in vegetative growth, abiscisic acid hypersensitivity, elevated tolerance to drought and oxidative stress (Xu et al. 2014). In Arabidopsis, Elongator subunit mutations lead to both up and down regulated gene expression (Zhou et al. 2009; Nelissen et al. 2010). The up regulation of transcripts may be a result from the indirect influence of down regulated genes. For example, Elongator positively regulates the expression of MYBL2, which encodes a single-repeat MYB protein and is a negative transcription factor in anthocyanin biosynthesis (Dubos et al. 2008). Therefore, *elo* mutants express higher levels of genes in the anthocyanin biosynthesis, and accumulate more anthocyanin than WT plants (Zhou et al. 2009).

Absorption of UV-B by DNA induces the formation of covalent bonds between adjacent pyrimidines, giving rise to cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts (Friedberg et al. 1995); overaccumulation of these products must be prevented to maintain genome

integrity. Therefore, plants have DNA repair systems to remove DNA lesions (Hays 2002; Bray and West 2005; Kimura and Sakaguchi 2006). In our experiments, *hag3* RNAi transgenic plants showed less CPDs than Ws plants after a UV-B treatment, while *hag1* and *hag2* deficient plants showed similar DNA damage as WT plants. Interestingly, transcripts for one photolyase, *UVR2*, and one subunit of the NER DNA repair complex, *UVR7*, were highly expressed in *hag3* plants even under control conditions in the absence of UV-B, suggesting that the lower accumulation of photoproducts by UV-B may be due to increased DNA repair in these mutants.

Plants have also evolved mechanisms that filter or absorb UV-B to protect against DNA damage (Mazza et al. 2000; Bieza and Lois 2001). *hag3* RNAi plants showed increased accumulation of UV-B absorbing pigments and anthocyanins, this phenotype was already reported for other *elo* mutants (Zhou et al. 2009). Therefore, it is also possible that the decreased levels of DNA damage after UV-B exposure may be due to either or both increased DNA repair or/and high production of UV absorbing products. Moreover, transcript levels of *HY5* are also increased in *hag3* plants, this transcription factor is a master regulator of many UV-B regulated transcripts. Thus, other and different mechanisms of UV-B protection may be also increased in *hag3* RNAi plants. In this respect, other *elo* mutants were found to be more tolerant to oxidative reagent methyl-viologen and H_2O_2 (Chen et al. 2006; Zhou et al. 2009), and the *elp1* mutant was also more drought tolerant than wild type (Chen et al. 2006). Interestingly, leaf stomata closure and root elongation of *elp1* and *elp2* mutants were more sensitive to plant hormone ABA (Chen et al. 2006; Zhou et al. 2009); and free auxin, ethylene, and jasmonic acid content in Elongator mutants were higher than that in wild type (Zhou et al. 2009). Therefore, the hormone crosstalk and the secondary effect of hormone imbalance on gene expression may also partially explain the phenotypes previously described of these mutants in abiotic stress (Nelissen et al. 2010) and now in UV-B tolerance.

The higher CPD repair in *hag3* plants correlates with lower plant and primary root growth inhibition by UV-B, demonstrating again that these plants have higher UV-B tolerance than WT plants. We found that *hag3* plants showed a smaller rosette and leaf area and retarded primary root growth in the absence of UV-B, as it was previously described (Nelissen et al. 2003 and 2005). Different reports indicated that mutations of Elongator subunits in Arabidopsis have pleiotropic effects on plant growth and development (Nelissen et al. 2005; Chen et al. 2006). However, after the UV-B treatment, final rosette and leaf area, and root length was similar in the transgenic and WT plants. Interestingly, palisade cell number in leaf #5, which was in actively cell division at the moment of the UV-B treatment, was similar as

in WT leaf #5 under control conditions in the absence of UV-B, while average cell area was smaller in the mutants. This is in contrast to what was previously described for this and other *elo* mutants, which showed a significant decrease in leaf cell number (Nelissen et al. 2005). However, and according to our results, while the decrease in cell number was observed in different cell types and at different developmental stages (Nelissen et al. 2005; Falcone *et al.* 2007); in particular developmental stages, the number of cells in some and particular leaves of *elo* mutants was not different in comparison to that in WT plants, while cell area was decreased (Falcone et al. 2007). After the UV-B treatment, both WT and *hag3* mutants showed a significant and similar inhibition of cell proliferation as it was previously reported (Casadevall et al. 2013). Nevertheless, while average cell area was not affected by the UV-B treatment in Ws plants, the *hag3* RNAi plants showed a significant increase in cell area, reaching a cell size similar to that of WT leaves. This "compensation" effect after the UV-B treatment may occur so that leaf #5 from the *hag3* RNAi plants, which already has a cell proliferation defect, can reach a final leaf size and it is not further reduced. In this way, after a UV-B treatment and in the *hag3* plants, the reduction in leaf cell number would trigger the induction of cell expansion. The compensation effect that we propose here, where a deficiency of cell proliferation triggers cell expansion, has been reported several times, and was recently reviewed by Horiguchi and Tsukaya (2011). As levels of the miR396 and GRFs, which we previously showed to participate in the inhibition of cell proliferation by UV-B (Casadevall et al. 2013) were not affected in *hag3* plants, the mechanism(s) by which HAG3 regulate plant growth inhibition by UV-B remain(s) to be investigated. On the other hand, while *hag1* and *hag2* plants were smaller than WT plants under control conditions in the absence of UV-B, these plants did not show differences in plant and root growth inhibition after UV-B irradiation with WT plants.

It is important to note that all the experiments presented in this manuscript were done using *hag3* RNAi lines, which have reduced levels of *HAG3* transcript expression and are not knockout lines. It would be interesting to confirm our results using knockout mutants; however, despite we screened for two different knockout T-DNA mutants lines, we were not able to indentify any knockout mutant after screening 60 seeds of the GK-555H06 line; while homozygous seedlings of the SALK_104121 line (with a T-DNA insertion in the 3'UTR) did not show decreased expression levels of *HAG3*.

Interestingly, our data demonstrates that, although *hag1* and *hag2* plants have similar phenotypes as WT plants after UV-B exposure, some UV-B regulated transcripts show a different pattern of expression in these lines in comparison to WT plants. HAG1, HAG2 and HAG3 only have in common the presence of a HAT

domain, while they have low sequence similarity and they have been shown to acetylate histones in different Lys residues (Supplementary Fig. S1; Earley et al. 2006 and 2007; Xu et al. 2012). In addition, these three proteins were reported to have different functions in Arabidopsis (Sterner and Berger 2000; Servet et al. 2010; Nelissen et al. 2005; Chen et al. 2006 ; Zhou et al. 2009, Defraia et al. 2010; Xu et al. 2012; Ramirez-Parra et al. 2003; Vandepoele et al. 2005). Therefore, it is possible that HAG1 and HAG2 may also participate in some but different aspects of UV-B responses in Arabidopsis than those mediated by HAG3, according to their different structure and function.

In summary, our results demonstrate that HAG3 negatively regulates the expression of genes that participate in UV-B responses in Arabidopsis, such as DNA repair enzymes and enzymes that participate in sunscreen pigment biosynthesis. Thus, increased levels of UV-B absorbing compounds, high expression of DNA repair enzymes, resistance against oxidative stress and the hormone crosstalk and imbalance affecting the expression important in UV-B responses may be the causes of *hag3* RNAi plants increased tolerance. However, other roles of the Elongator complex described in different species, such as tRNA nucleoside modification (Huang et al. 2005; Esberg et al. 2006), transcriptional silencing (Li et al. 2009), tubulin acetylation (Creppe et al. 2009), and/or even a direct participation in DNA damage repair (Li et al. 2009) may also be affecting the tolerance phenotype of *hag3* mutants. More experiments are needed to demonstrate which function(s) of this complex is (are) involved in UV-B responses.

Materials and Methods

Plant material, growth conditions and irradiation protocols

A. thaliana ecotype Columbia (Col-0) and Wassilewskija (Ws-2) lines were used for the experiments. The RNAi transgenic lines (for *hag1*: CS30927, CS30928, CS30929 and CS30930; and for *hag3*: CS3981, CS3982 and CS3983) and the T-DNA insertion mutants (SALK_152796C and SALK_051832C) were obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH). The T-DNA insertion mutants were obtained from the SALK T-DNA insertion mutant collection. All plant lines used shown decreased *HAG* transcript levels as shown in Supplementary Fig. S3. Arabidopsis plants were sown directly on soil and placed at 4ºC in the dark. After 3 days, pots were transferred to a greenhouse and plants were grown at 22ºC under a 16h/8h light/dark regime. UV treatments were carried out in a growth

chamber with supplemental visible lighting to 100 $\mu Em^{-2}s^{-1}$ with 16 h of light and 8 h of dark. Plants were illuminated using UV-B lamps for 4 h (2 W/m^{-2} UV-B and 65 W/m^{-2} UV-A, Bio-Rad, Hercules, California) using fixtures mounted 30 cm above the plants. This UV-B flux rate corresponds to UV-B on 21 December at Rosario, Argentina. The bulbs were covered with cellulose acetate filters (CA, 100 mm extra-clear cellulose acetate plastic, Tap Plastics, Mountain View, CA); the CA sheeting does not remove any UV-B radiation from the spectrum but excludes wavelengths lower than 280 nm (UV-C). As a control of no UV-B, plants were exposed for the same period of time under the same lamps covered with polyester filters (PE, 100 mm clear polyester plastic; Tap Plastics). This PE filter absorbs UV-B. The output of the UV-B source were recorded using UV-B/UV-A radiometer (UV203 AþB radiometer; Macam Photometrics) to ensure that both the bulbs and filters provided the designated UV light dosage in all treatments. Samples were collected immediately after irradiation.

Identification of insertional T-DNA mutants

The genotype of plants with a T-DNA insertion was determined using a PCR-based approach. Initial screening was performed using genomic DNA isolated from leaves by a modified cetyl-trimetyl-ammonium bromide (CTAB) method (Sambrook & Russel, 2001) and three combinations of primers. Two primers hybridize to specific genomic sequences (Table 1) and one primer is located inside the left border of the T-DNA. The presence or absence of the T-DNA insertion in the genes allowed the identification of homozygous, heterozygous and WT plants.

Quantitative RT-PCR

Total RNA was isolated from about 100 mg of tissue using the TRIzol reagent (Invitrogen, Carlsbad, CA) as described by the Manufacture's Protocol. The RNA was incubated with RNase-free DNase I (1 U/ml) following the protocol provided by the manufacturer to remove possible genomic DNA. Then, RNA was reverse-transcribed into first-strand cDNA using SuperScript II reverse transcriptase (Invitrogen) and oligo-dT as a primer. The resultant cDNA was used as a template for qPCR amplification in a MiniOPTICON2 apparatus (Bio-Rad), using the intercalation dye SYBRGreen I (Invitrogen) as a fluorescent reporter and Platinum Taq Polymerase (Invitrogen). Primers for each of the genes under

study were designed using the PRIMER3 software (Rozen and Skaletsky 2000) in order to amplify unique 150-250 bp products (Table 1). Amplification conditions were carried out under the following conditions: 2 min denaturation at 94°C; 40 cycles at 94°C for 15 s, 57°C for 20 s, and 72°C for 20 s, followed by 10 min extension at 72°C. Three replicates were performed for each sample. Melting curves for each PCR were determined by measuring the decrease of fluorescence with increasing temperature (from 65ºC to 95ºC). PCR products were run on a 2% (w/v) agarose gel to confirm the size of the amplification products and to verify the presence of a unique PCR product. Gene expressions were normalized to the *A. thaliana* calcium dependent protein kinase3 (*CPK3*, Table 1). The expression of this gene has been previously reported to remain unchanged by UV-B (Ulm et al. 2004).

DNA Damage Analysis

The induction of CPD was determined using an assay described in detail previously (Stapleton et al. 1993). Monoclonal antibodies specific to CPDs (TDM-2) were from Cosmo Bio Co., Ltd. (Japan). After treatment, plant samples (0.1 g) were collected and immediately immersed in liquid nitrogen and stored at -80ºC. The 1.5 μg of the extracted DNA by a modified cetyl-trimetyl-ammonium bromide (CTAB) method was denatured in 0.3 M NaOH for 10 min and six-fold dot blotted onto a nylon membrane (Perkin Elmer life Sciences, Inc.). The membrane was incubated for 2h at 80ºC and then it was blocked in TBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) containing 5% dried milk for 1 h at room temperature or overnight at 4ºC. The blot was then washed with TBS and incubated with TDM-2 (1:2000 in TBS) overnight at 4ºC with agitation. Unbound antibody was washed away and secondary antibody (BioRad) conjugated to alkaline phosphatase (1:3000) was added. The blot was then washed several times followed by the addition of the detection reagents NBT and BCIP. Quantification was achieved by densitometry of the dot blot using ImageQuant software version 5.2. DNA concentration was fluormetrically determined using the Qubit dsDNA assay kit (Invitrogen), and checked in a 1% (w/v) agarose gels after quantification.

Rosette area quantification

Approximately 20 seeds per tray were sown, leaving enough space in between them to avoid superposition during plant growth. 12 DAS, a group of plants were subjected to a 4 h UV-B treatment (2

W m^{-2}) and another group was kept as control plants; after the treatment, all the plants were kept in a growth chamber until the end of the experiment. Every 3 days, photographs were taken and total leaf or rosette area of each plant was measured using the ImageJ software.

Microscopic observations

Leaves were fixed with FAA and cleared with chloral hydrate solution (200 g chloral hydrate, 20 g glycerol, and 50 ml dH_2O) as described (Horiguchi et al. 2005), and silhouettes of leaf images were acquired through a differential interference contrast (DIC). Leaf area was quantified using ImageJ image analysis software. Palisade leaf cells were observed by DIC microscopy, the area of palisade cells was determined, and the area of the leaf blade was divided by this value to calculate the total number of palisade cells in the subepidermal layer. To determine the cell area, 20 palisade cells were measured in each leaf. Experiments were carried out in duplicate with at least 10 leaves, obtaining similar results.

Root length measurements

Petri dish-grown seedlings, surface-sterilized seeds were grown on MS growth medium and were held vertical in a growth chamber. Then, seedlings were UV-B treated for 1h (4W m^{-2}) and kept in the absence of UV-B for 3 days Plates were photographed before the treatment, and 1, 2, 3 and 4 days after the end of the treatment, and the images were analyzed using the ImageJ program. Root lengths were determined by measuring the length of a line traced along the root.

Pigment measurements

UV-absorbing pigments (absorbance at 312 and 530 nm) were determined as described in Casati and Walbot (2004).

Statistical analysis

Statistical analysis was done using ANOVA models (Tukey test) or alternatively Student's t test, using untransformed data.

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Table 1. Primers used for PCR.

Name	Sequence
T-DNA left border	5′-GTCCGCAATGTGTTATTAAGTTGTC-3′
SALK_051832 L	5′-TGTAAAGTTCGCCGGAAA-3′
SALK_051832 R	5′-AATTTTCCCGCCTTTGTC-3′
HAG1 L	5′-GTGGATTCTCGCGATGTC-3′
HAG1 R	5′-TCGGGGGAGTTGTAAGTTC-3′
HAG2 L	5′-GCGTTTGACCACTCTTGG-3′
HAG2 R	5′-AGCCAGAAGCATCGTTTG-3′
HAG3 L	5′-TCGGTGTGATTTCTGGTGT-3′
HAG3 R	5′-CGCCATAGTTCGTGAAGG-3′
UVR2 F	5′-GACCCGAGTGGATATGTTGG-3′
UVR2 R	5′-GAGCTGTTCTTCAGCTTTCC-3′
UVR7 F	5′-TACATTCGGGTCTCTTGCTC-3′
UVR7 R	5′-TCCTCGTCTTCTTCAACAGG-3′
HY5 F	5′-TCCTTTTCACCAGCTTCG-3′
HY5 R	5′-TTTTCCGACAGCTTCTCC-3′
CHS F	5′-TGATGGCTGGTGCTTCTT-3′
CHS R	5′-GACGTTTCCGAATTGTCG-3′
F3H F	5′-GGAAGAGATTTGGAGCTTGC-3′
F3H R	5′-CACACCGAGCCTAGCATAAT-3′
CPK3 F	5′-ATCTGGAGTGCTGGTGTGAT-3′
CPK3 F	5′-AATCCACGGATGATTTAGCA-3′

FIGURE LEGENDS

Fig. 1. Plant growth inhibition by UV-B in *hag1, hag2* and *hag3* plants. WT (Ws and Col0) and *hag1, hag2* and *hag3* plants were treated with UV-B radiation for 4 h (2 W m^{-2}, right) or were kept under conditions in the absence of UV-B. (A, C and E) Rosette area of control and UV-B treated Ws and *hag1* (A), Col0 and *hag2* (C), and Ws and *hag3* (E) plants measured every 3 days from germination until 21 DAS. Plants were UV-B treated 12 DAS (indicated with an arrow). (B, D and F) The ratio of rosette areas of UV-B treated vs control plants for each line is shown. Results represent the average of 10 biological replicates ± S.E.M. Asterisks denote statistical differences applying Student's t test ($P < 0.05$). (G) Representative picture of Ws and *hag3* RNAi plants treated with UV-B radiation or kept under conditions in the absence of UV-B nine days after the end of the treatment.

Fig. 2. UV-B effect in leaf development in *hag3* RNAi plants. Relative average leaf area (A), estimated cell number (B), cell area (C), and cell length vs cell width ratio (D) of fully expanded leaf #5 from UV-B treated versus control Ws and *hag3* (CS3981) Arabidopsis plants. Results represent the average of 10 biological replicates ± S.E.M. Statistical significance was analyzed using ANOVA, Tukey test with $P < 0.05$; differences from the control are marked with different letters.

Fig. 3. Primary root inhibition assays in WT and *hag1, hag2* and *hag3* plants after UV-B exposure. (A, C and E) Graph of average root lengths in Ws and *hag1* plants (A), Col0 and *hag2* plants (C), and Ws and *hag3* plants (E) up to 4 days after a UV-B treatment or under control conditions in the absence of UV-B (C). Statistical significance was analyzed using ANOVA, Tukey test with $P < 0.05$; differences from the control are marked with different letters. The average root lengths after UV-B exposure relative to the length in control seedlings is shown in B, D and F. Asterisks denote statistical differences applying Student's t test ($P < 0.05$). Results represent the average of 20 biological replicates ± S.E.M. (G) Representative picture of Ws and *hag3* plants treated with UV-B radiation for 1 h or kept under conditions in the absence of UV-B 4 days after the end of the treatment.

Fig. 4. UV-B absorbing compounds and anthocyanin levels in WT and *hag1, hag2* and *hag3* plants after UV-B exposure. Total UV-B absorbing compounds, were assayed after 4h UV-B (UV-B) compared to untreated controls (C) in Ws plants and *hag1* (A), Col0 and *hag2* (B), and Ws and *hag3* plants (C). The ratio of pigment absorbance at 312 nm of UV-B treated vs control plants for each line is shown in E-G. Anthocyanins, determined by pigment absorbance at 530 nm, were assayed after 4h UV-B (UV-B) compared to untreated controls (C) in Ws and *hag3* plants (D). Measurements are the average of six adult leaves from six different plants. Error bars represent S.E.M. Statistical significance was analyzed using ANOVA, Tukey test with $P < 0.05$; differences from the control are marked with different letters

Fig. 5. CPD levels in the DNA of WT and *hag1, hag2* and *hag3* plants after UV-B exposure. CPD levels in DNA of UV-B treated Ws and *hag1* (A), Col0 and *hag2* (B), and Ws and *hag3* (C) plants for 4h. Experiments were done under conditions that allowed photorepair in the light. 1.5 μg of DNA was loaded in each well. Results represent the average ± S.E.M. of six independent biological replicates. Asterisks denote statistical differences applying Student's t test ($P < 0.05$).

Fig. 6. Relative transcript levels of UV-B regulated transcripts in WT, *hag1* (A), *hag2* (B), and *hag3* (C) plants measured by qRT-PCR. Plants were irradiated with UV-B light for 4h (UV-B) or kept under control conditions (C). Data show mean values ± S.E.M of at least three independent experiments. Statistical significance was analyzed using ANOVA, Tukey test with $P < 0.05$.

Supporting information

Supplementary Fig. S1. Amino acid sequences and schematic view of *A. thaliana* GNAT family transcripts. (A) HAG1, (B) HAG2, and (C) HAG3. Blue boxes represent exons, thin black lines represent introns, and grey boxes the UTR regions. The HAT and other domains are shown in violet.

Supplementary Fig. S2. Relative expression of *HAG1, HAG2* and *HAG3* transcripts by RT-qPCR. Col0 Arabidopsis plants were irradiated with UV-B for 4 h (UV-B) or were kept under control conditions without UV-B (C). Expression values are relative to the *CPK3* control. Data show mean values ± S.E.M. of at least three independent experiments.

Supplementary Fig. S3. Relative transcript levels of *A. thaliana HAG1*, *HAG2* and *HAG3* genes measured by qRT-PCR in WT plants and in *hag1* (A) and *hag3* (C) RNAi transgenic plants, and in a *hag2* T-DNA insertional mutants (B). The location of the T-DNA insertions in the *HAG2* gene is shown in (B). Exons are represented by blue boxes, introns by thin black lines and the UTR regions by light grey boxes. The T-DNA insertion is indicated as a triangle.

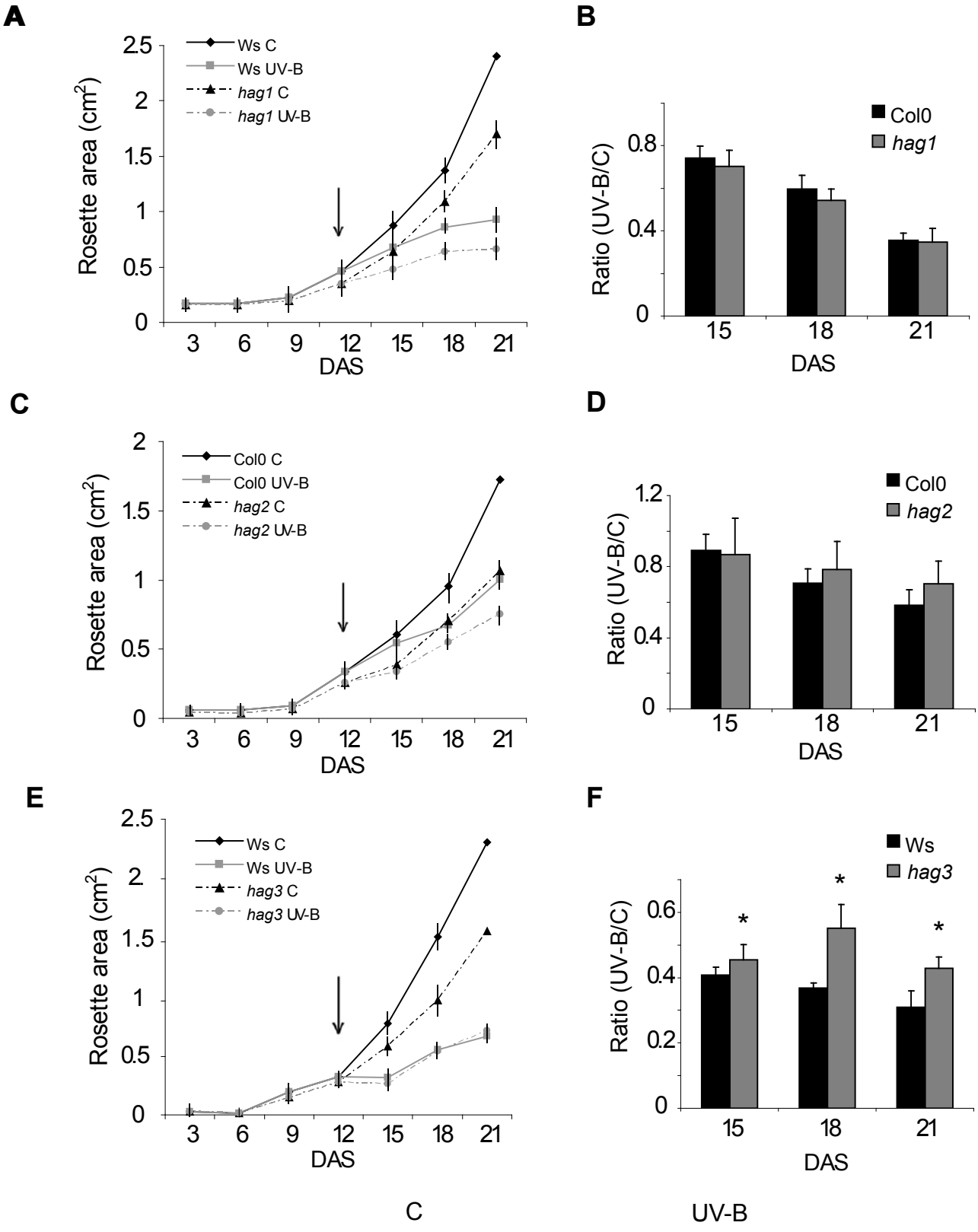

Fig. 1

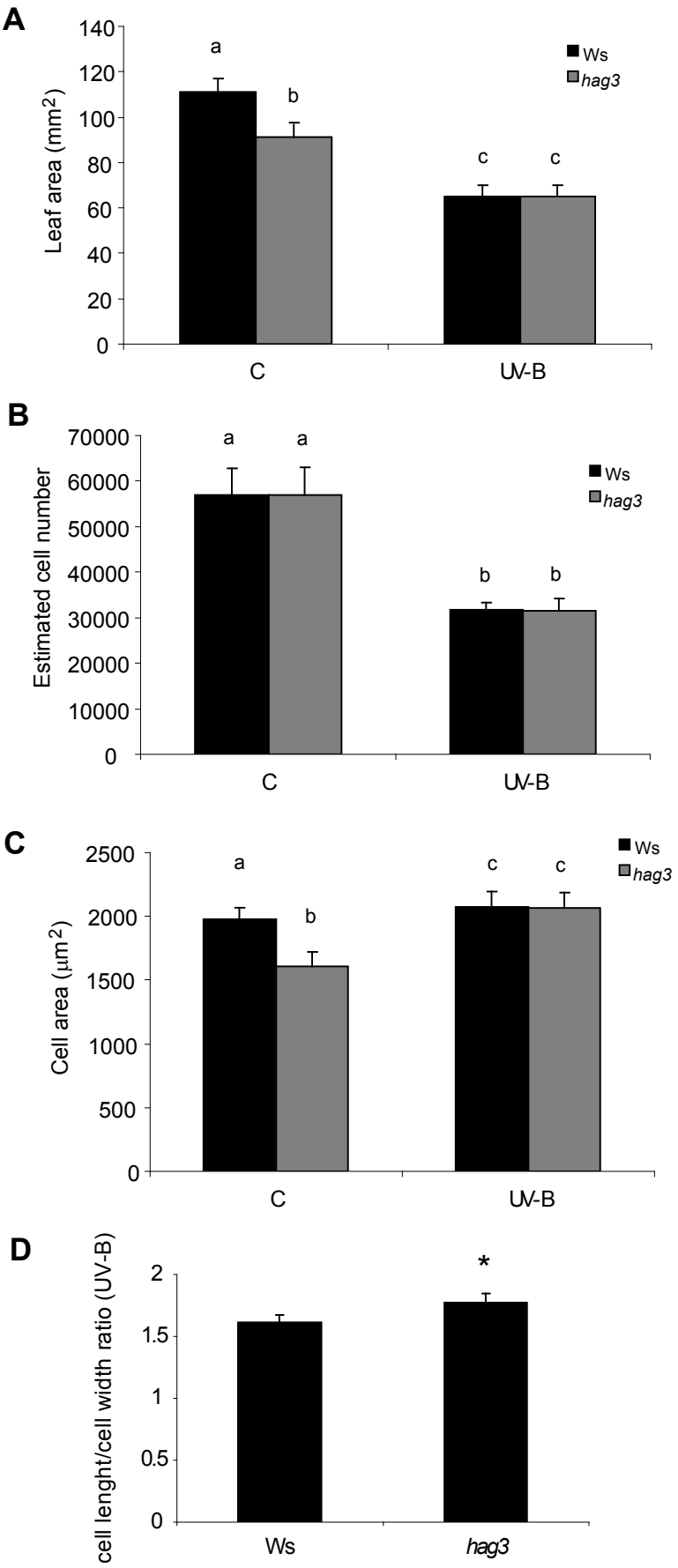

Fig. 2

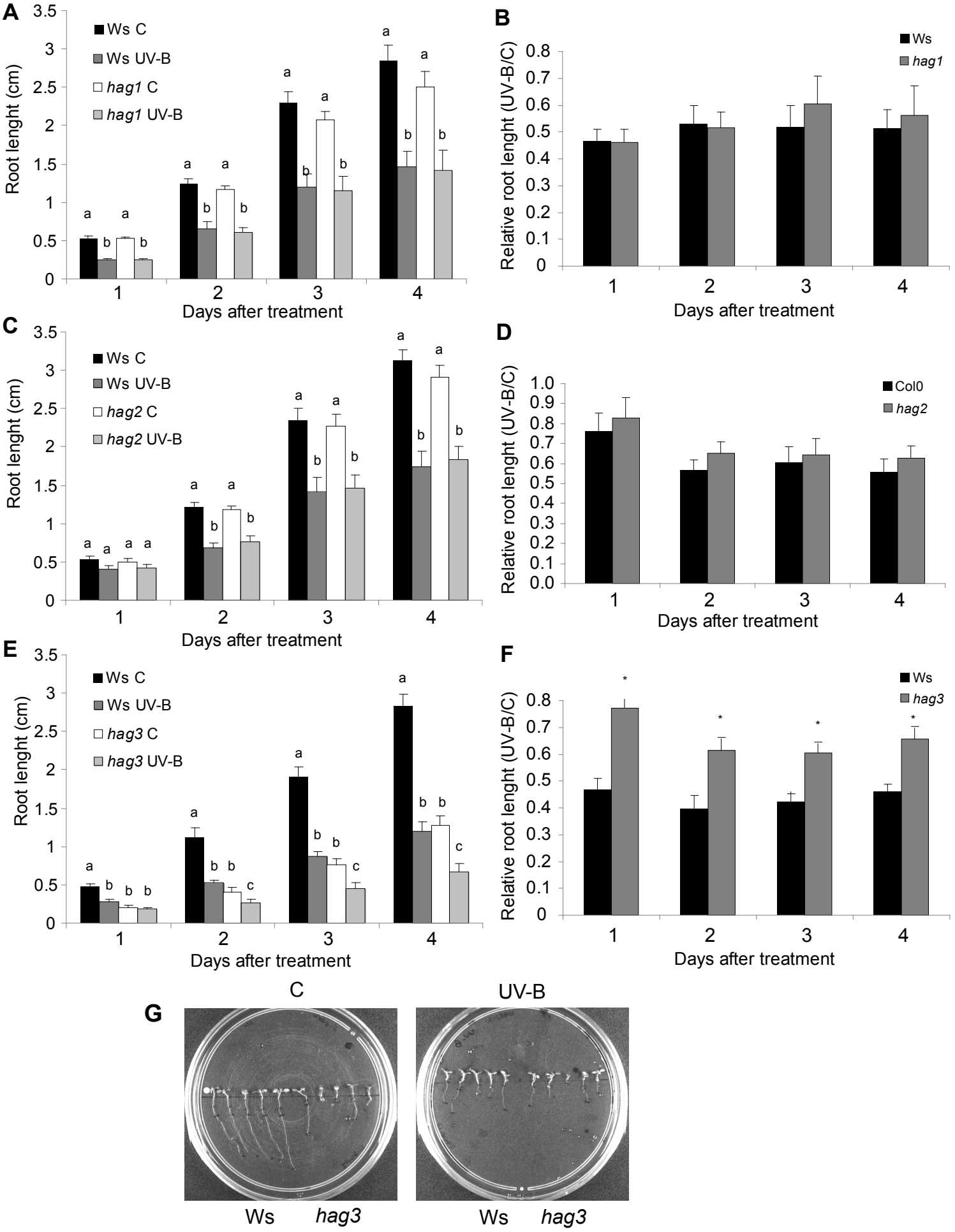

Fig. 3

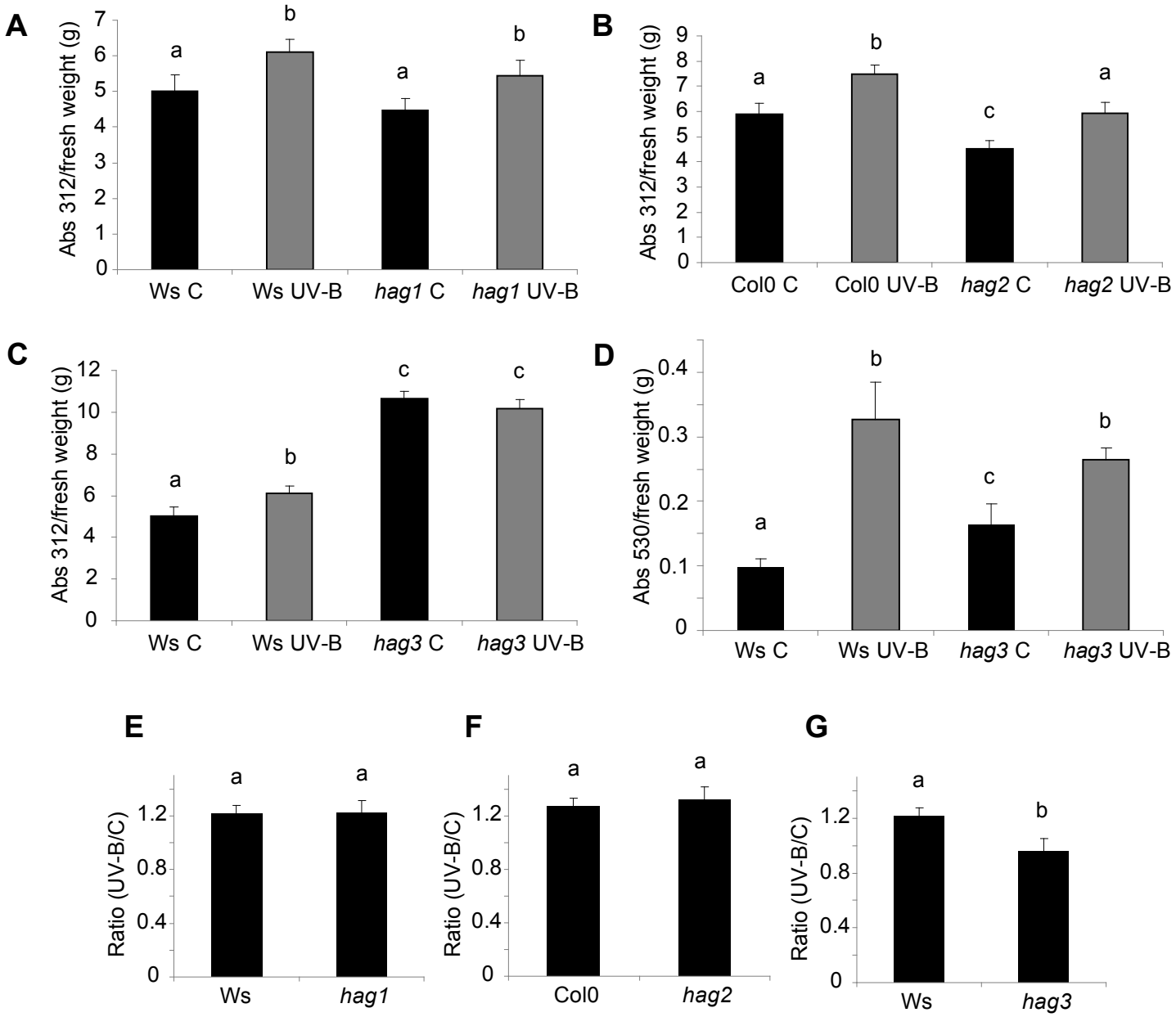

Fig. 4

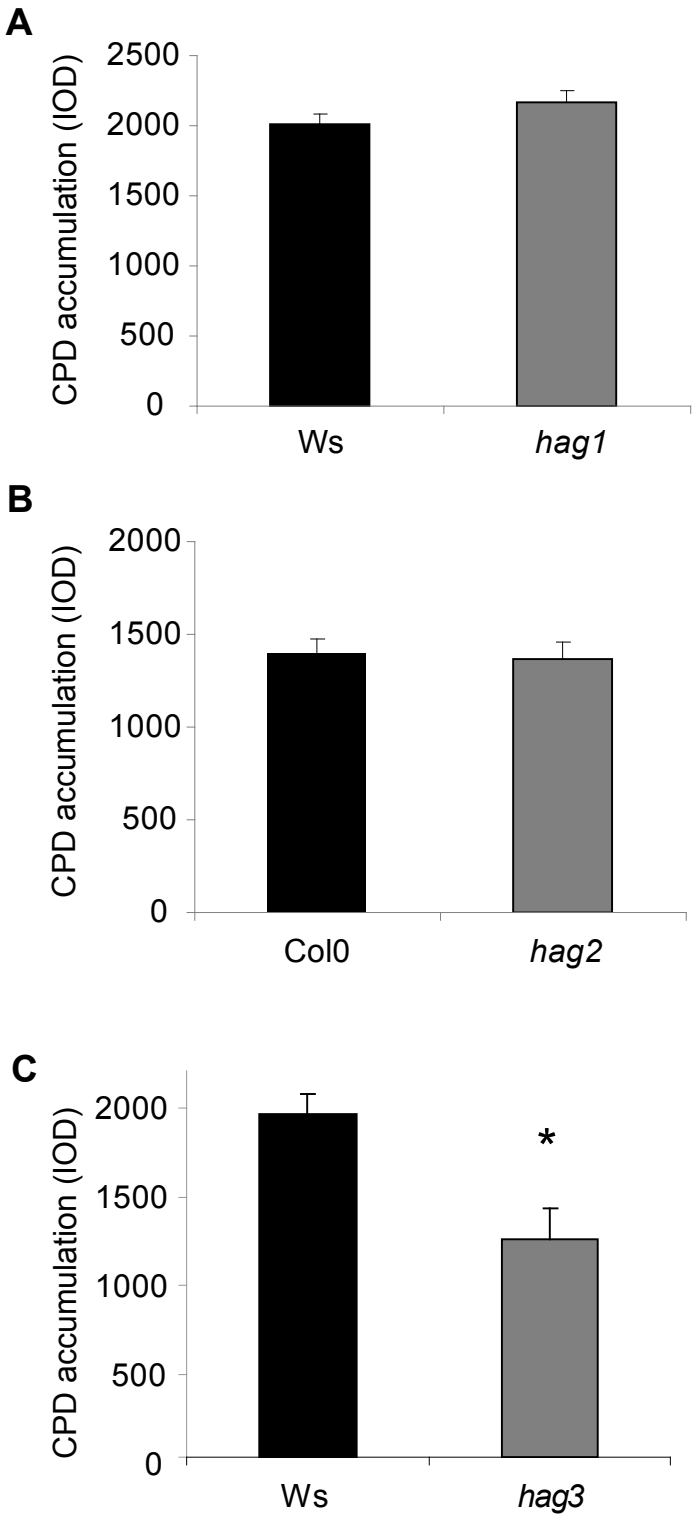

Fig. 5

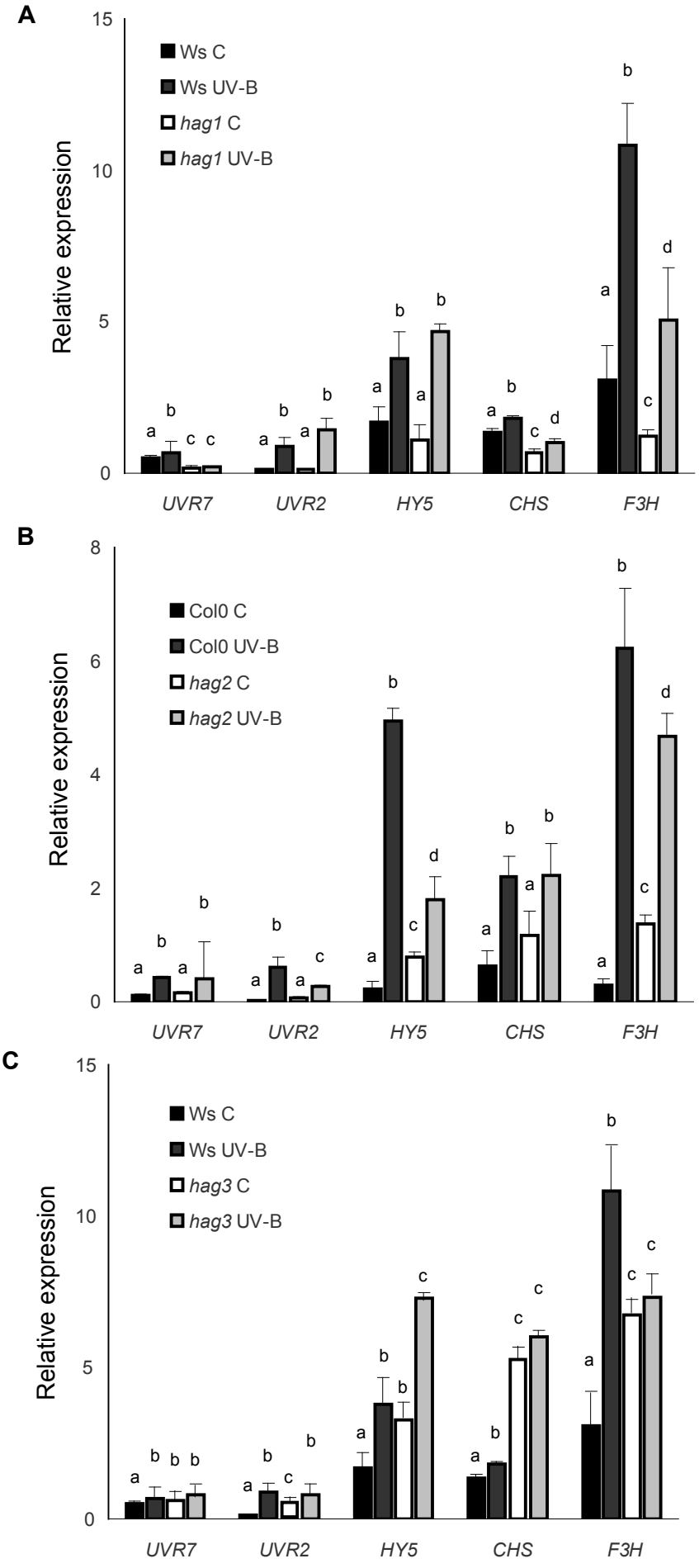

Fig. 6